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P nucleotide insertions and the resolution of hairpin DNA structures in mammalian cells

[V(D)J recombination/end joining/illegitimate recombination/severe combined immunodeficiency/*scid*]

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ABSTRACT **Two lines of evidence point to a hairpin DNA intermediate in V(D)J joining (V, variable; D, diversity; J, joining) [Lieber, M. R. (1991) *FASEB J.* 4, 2934–2944]. One is the presence of P nucleotide insertions (short inverted-repeat sequence) in V(D)J junctions [Lafaille, J. J., DeCloux, A., Bonneville, M., Takagaki, Y. & Tonegawa, S. (1989) *Cell* 59, 859–870]; a second is the detection of site-specifically broken DNA molecules with covalently closed (hairpin) termini in thymus DNA [Roth, D. B., Menetski, J. P., Nakajima, P., Bosma, M. J. & Gellert, M. (1989) *Cell* 70, 983–991]. However, P nucleotide insertions could be generated in ways not involving a hairpin structure, and because physical evidence for hairpin-ended DNA fragments has been obtained only with mutant mice, there is some uncertainty regarding the role of hairpin molecules in the normal V(D)J joining pathway. To determine whether mammalian cells are capable of metabolizing this odd type of DNA terminus and whether, in doing so, junctions with P insertions are in fact created, a linear DNA molecule with a hairpin closure at each end was transfected into several murine cell lines. The hairpin-ended molecules were recircularized, and the junctions exhibited P insertions at a high frequency. This result directly links the presence of P insertions to a hairpin precursor, providing strong evidence for the notion that a hairpin DNA intermediate exists in V(D)J recombination. A comparison of hairpin end joining in various cells, including those derived from mice with the severe combined immunodeficiency (*scid*) mutation, is presented.**

Two types of insertion are found in V(D)J junctions (V, variable; D, diversity; J, joining) (reviewed in ref. 1). Each provides a clue to the enzymatic operations accomplishing antigen receptor gene assembly in B and T cells. One type of insertion, random in sequence, is termed an N region and has been shown to be due to the activity of terminal deoxynucleotidyltransferase in rearranging lymphoid cells (2, 3). A second type, termed P insert, occurs adjacent to nontruncated V, D, or J segments (ref. 4; reviewed in ref. 1) and constitutes a very short inverted repeat of the adjacent full-length gene segment. Although infrequent (occurring in <10% of endogenous junctions), these "palindromic" insertions have been demonstrated with artificial joining constructs (5) and where terminal deoxynucleotidyltransferase activity is absent (2, 3). Similar junctional inserts have been observed in the case of plant transposons where, upon exit of the transposable element, a resealed genomic site sometimes exhibits inverted repeat DNA sequence (6, 7). Various mechanisms have been suggested to account for the presence of short inverted repeats at junctions—for example, template switching by a DNA polymerase (6), direct transfer of a block of nucleotides from one cut DNA strand to the other (5), or generation and breakdown of a hairpin DNA intermediate (7, 8).

In the case of V(D)J joining, there is some direct evidence for a hairpin structure: thymus DNA preparations contain cleaved molecules sealed by a 5′-to-3′ closure at the tip (9). As demonstrated by Southern blot hybridization analyses, the covalently closed breaks were mapped to specific recombination sites within the T-cell receptor δ locus (9). One intriguing aspect of the study was that the covalently sealed ends were readily demonstrated in *scid* but not in wild-type samples (9). The severe combined immunodeficiency associated with this mutation in mice has been traced to ineffective V(D)J joint formation (10), but *scid* cells also exhibit joining defects of a more general nature. Both lymphoid and nonlymphoid lineages are hypersensitive to DNA-damaging agents, particularly those creating double-strand breaks (11–13). An hypothesis that unifies the V(D)J joining and DNA-repair phenotypes in *scid* is that an endonuclease required for hairpin resolution in V(D)J joining is defective in *scid* animals (9). Although critical for antigen receptor gene assembly, the endonuclease would also play a role in certain modes of DNA repair, explaining the pleiotropic effects of the *scid* mutation. Accordingly, a putative "hairpin nick-ase" would be expected to have a wide tissue distribution.

To determine whether hairpin DNA is in fact a metabolizable substrate in eukaryotic cells, and, if so, to investigate the relationship between hairpin processing and P nucleotide insertion, a linear DNA with covalently closed termini (Fig. 1) was assembled *in vitro* and then transfected into murine cell lines. The ability to join the hairpin ends of the linear molecule, generating a circular plasmid, was tested and the hairpin resolution junctions were characterized. Four questions were posed. (*i*) Do cells possess necessary activities required to achieve hairpin end joining? (*ii*) If so, does this process result in the introduction of P nucleotide inserts within the product junctions? (*iii*) Is hairpin end-joining activity limited to cells that carry out V(D)J recombination? (*iv*) Is the hairpin resolution process defective or otherwise variant in *scid*?

MATERIALS AND METHODS

Preparation of the Hairpin Substrate. The shuttle vector pJH298 (14); was doubly digested with *Bam*HI and *Sal* I. The large 7.7-kb fragment was gel purified. Fifty micrograms of this DNA was ligated with T4 DNA ligase. After heat inactivation at 65°C for 1 hr, the preparation was incubated with *Aat* 2 (small arrows in Fig. 2 represent *Aat* 2 sites). Head-to-head dimers (with a central *Bam*HI or *Sal* I site) were isolated. The two purified DNA fragments were denatured by heating at 100°C for 4 min, the $MgCl_2$ concentration was adjusted to 10 mM, and samples were allowed to cool to room temperature. Self-annealing, which folds the fragments

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Abbreviations: V, variable; D, diversity; J, joining.

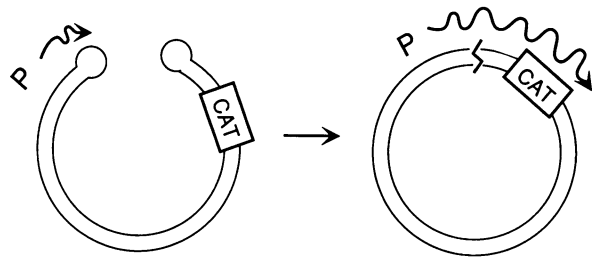

FIG. 1. Recircularization assay. P, promoter; CAT, chloramphenicol acetyltransferase gene. Other features of the substrate (those supporting bacterial and eukaryotic replication, and the β-lactamase gene with its promoter) are not shown. Hairpin linear DNA is transfected into murine lymphoid cells. Recircularized molecules confer chloramphenicol and/or ampicillin resistance upon introduction into *E. coli*.

to half their previous length, was verified on analytical gels. Folded molecules were mixed together and ligated at the compatible *Aat* 2-cleaved ends created at their open termini. The 7.7-kb linear DNA corresponding to the desired product (Fig. 2, boxed) was gel purified. DNA concentration was estimated by agarose gel electrophoresis against known standards.

Cell Lines and Transfection Assay. Abelson murine leukemia virus transformed cell lines 204-1-8 (wild type) and S41 (*scid*) have been described (15, 16). NIH 3T3 cells were obtained from S. Birren (California Institute of Technology). The identity of the S41 cell line was verified by transfection of V(D)J recombination substrates (data not shown). DEAE-dextran-mediated transfection of DNAs into lymphoid cell lines and harvest of the transfected DNA were as described (16), except that quantities listed in Table 2 were used. In some experiments, an unrelated ampicillin-resistant plasmid lacking eukaryotic replication capability (carrier) was included with the hairpin DNA (details furnished upon request). DEAE-dextran-mediated transfection of NIH 3T3 cells was as described (ref. 17, protocol 1); treatment with a DEAE-dextran solution (1 mg/ml) was for 6 min only. Electrotransformable and competent DH10b cells were either purchased or prepared according to protocols supplied by Bethesda Research Laboratories. Transformations were according to the manufacturer's specifications, with the exception that 20-μl aliquots of the bacterial cells were used. Transformants were selected on plates containing chloramphenicol (10 μg/ml) and/or ampicillin (100 μg/ml) and restreaked to plates containing chloramphenicol (40 μg/ml). Those that grew on the higher chloramphenicol concentration (whether or not growth was robust) were randomly selected for DNA sequence analysis without further screening. In some cases, aliquots of transfected samples were digested with *Dpn* I before *Escherichia coli* transformation (16). (*Dpn* I digests DNA that has not undergone eukaryotic replication, eliminating the ampicillin-resistant carrier DNA without reducing the number of recovered chloramphenicol-resistant isolates; data not shown). In early experiments, recircularized molecules were isolated that included partial duplications, large deletions, and inserts of >100 bp. These were extensively characterized; however, they proved atypical in that retransformation of the same transfected samples on a new lot of selective plates consistently yielded higher numbers of recombinants, and the randomly selected isolates from the later transformations all had a simple recircularized structure. The DNA sequence of the more highly rearranged molecules was determined (data not shown), and, despite their more scrambled structure, the frequency and length of P insertions at full-length ends was no different than evidenced by the retransformed samples (Fig. 3, isolates 101–114). Nor were there any differences between wild-type and *scid* collections. Data from these analyses were thus included in the totals shown in Table 2 (columns 4–6).

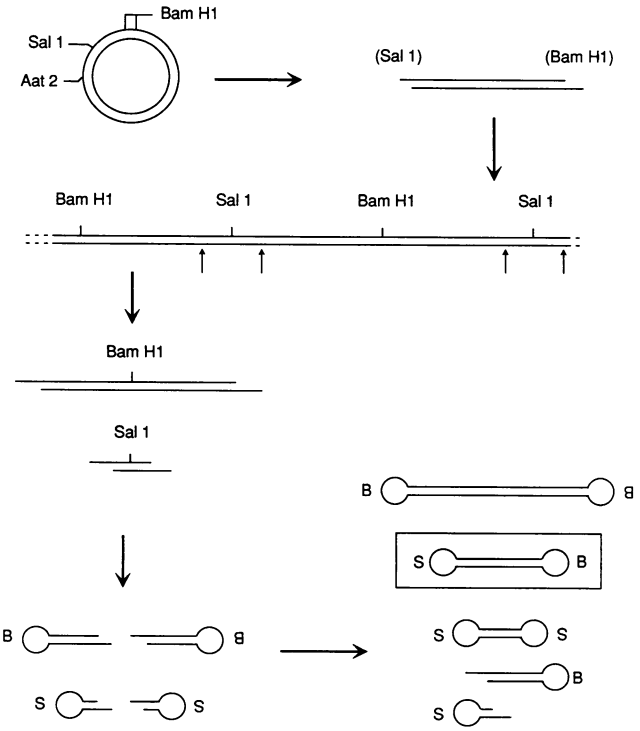

FIG. 2. Preparation of the hairpin substrate. Illustration is not to scale. All *Bam*HI, *Sal* I, and *Aat* 2 sites are shown; B or S at the terminal bubbles, *Bam*HI or *Sal* I recognition sites after denaturation and self-annealing; corresponding restriction sites no longer exist within the loops.

DNA Sequence Determinations and Assignment of Residues Within Junctions. DNA sequence determination (Sanger method) was performed with four primers (sequences furnished upon request) that annealed at positions −26 and −108 on the left terminus and −16 or −144 residues from the right end. Junctions with identical sequences were listed (and scored) only where isolated from independent transfections. In Fig. 3, residues that might be assigned to either of two ends were written so that they would, if possible, comprise at least one full-length terminus (thus, a *maximum* value for full-length ends is provided). Residues that might belong either to an input end or to a P insert were always assigned to the end, not the insert. Likewise, where alternative assignments involved two P inserts, residues were assigned so as to provide *minimum* values for the number and length of P inserts.

Substrate Purity. Five nanograms of the hairpin DNA preparation did not result in any transformants when introduced directly into *E. coli*. The "parental" circular plasmid pJH298 DNA gave 3.2×10^4 colonies per ng, while the 7.7-kb *Sal* I/*Bam*HI linear DNA gave rise to 21 colonies per ng (relative values were confirmed by mixing experiments). On this basis, the hairpin preparation appeared to be free of open linear DNA. Further, there was no significant level of open-ended DNA in the hairpin preparations because (*i*) P inserts of four (or more) residues were readily observed (Fig. 3) and as such could not have been generated by filling in *Sal* I- or *Bam*HI-digested termini. (*ii*) P insert frequencies were virtually identical when different hairpin preparations were transfected (data not shown). (*iii*) The hairpin substrate was recircularized *more* efficiently in lymphoid cells than was the corresponding open linear DNA (Table 1).

GGCTGCAGGTC
CCTACGTCCAG

TCCCCGGGGAT
AGGGGCCCCTA

	LEFT SIDE	(P)		(P)	RIGHT SIDE
A.					
HPW-114	GGCTGCAGGTC				CGGGGAT
HPW-108	GGCTGCAGGTC	GA			CCGGGGAT
HPW-109	GGCTGCAGGTC	GA			TCCCCGGGGAT
HPW-132	GGCTGCAGGTC	GA			TCCCCGGGGAT
HPW-111	GGCTGCAG		CCC	A	TCCCCGGGGAT
HPW-113	GG			GGA	TCCCCGGGGAT
HPW-112	-17..(AGCT)				-16
HPW-133	-12				GGGGAT
HPW-134	GGCTGCAGG				-28
B.					
HPS-138	GGCTGCAGGTC	GA			TCCCCGGGGAT
HPS-139	GGCTGCAGGTC	GA	TTGT	GGA	TCCCCGGGGAT
HPS-103	GGCTGCAGGTC	GACCT		GA	TCCCCGGGGAT
HPS-107	GGCTGCAGG				TCCCCGGGGAT
HPS-101	GGCTGCAGG			A	TCCCCGGGGAT
HPS-105	GGCTGCA		ATCAT	GGA	TCCCCGGGGAT
HPS-104	-13		CGGGCGTA	A	TCCCCGGGGAT
HPS-137	GGCTGCAGG				AT
HPS-140	GGCTGCAGG		insert=28		-14
C.					
HPF-65	GGCTGCAGGTC				CCGGGGAT
HPF-71	GGCTGCAGGTC				CCGGGGAT
HPF-75	GGCTGCAGGTC	GA			CCGGGGAT
HPF-69	GGCTGCAGGTC				CCCGGGGAT
HPF-77	GGCTGCAGGTC				CCCGGGGAT
HPF-68	GGCTGCAGGTC	GA			CCCCGGGGAT
HPF-79	GGCTGCAGGTC	GAC			CCCCGGGGAT
HPF-61	GGCTGCAGGTC	GA			TCCCC-GGGAT
HPF-74	GGCTGCAGGTC	GA			TCCCCGGGGAT
HPF-66	GG			GGA	TCCCCCGGGAT
HPF-76	-26				TCCCCCGGGAT
HPF-62	GGCTGCAGG				GGAT
HPF-70	-11(G)				-21
HPF-72	-73(GG)				GAT
HPF-73	GGCTGCAG				-14
HPF-78	GGCTGCAGG				GGAT

FIG. 3. Sequences of junctions isolated with the hairpin substrate. One insert is indicated by size only. Underlining indicates residues that could be assigned to either of two ends. All junctions containing at least one nontruncated terminus are shown above the dashed line. Below the dashed line are listed all recombinants in which both left and right sides have been truncated. (*A*) Junctions isolated from wild-type cell line 204-1-8. (*B*) Junctions isolated from *scid* cell line S41. (*C*) Junctions isolated from NIH 3T3 cells.

RESULTS

A Hairpin Resolution Assay. To test cells for the ability to resolve hairpin DNA ends, a shuttle vector (14) was cut between its chloramphenicol acetyltransferase gene and the corresponding promoter. The termini of the resulting linear DNA preparation were sealed to form hairpin ends (Figs. 1 and 2). The manufacture of the linear hairpin-ended DNA and confirmation of its structure are detailed below. The hairpin substrate was transfected into several different cell lines, and DNA was harvested from the transfected cells ≈48 hr later. Successfully recircularized molecules were revealed upon transformation of *E. coli* to chloramphenicol and/or ampicillin resistance.

Hairpin Resolution in Wild-Type and *scid* Lymphoid Cells. The hairpin substrate was transfected into two lymphoid cell lines representative of early B-lineage cells. Recircularization was indicated by the fact that colonies arose after transformation of the transfected samples into *E. coli*. No such colonies arose without prior transfection (see below). About 60% of the isolates were resistant to both chloramphenicol and ampicillin (Table 2). The locations of the β-lactamase and chloramphenicol acetyltransferase genes relative to the hairpin ends were such that ≈10 times more sequence was dispensible for ampicillin than for chloramphenicol resistance. Thus, the high fraction of doubly resistant molecules indicated that nonspecific breakage, one possible means of hairpin removal, was not the principal mechanism. Of particular note was the fact that the chloramphenicol-resistant molecules, which are the focus of the remainder of the study, represented the majority population.

Table 1. Recircularization of DNAs: *scid* (S41) vs. wild type (1-8)

Cell line	DNA	Amount,* ng	Cam^r/50†
1-8	Hairpin (1) + carrier	20–40	161
1-8	Hairpin (3)	10	10
1-8	Hairpin (3)	10	0
1-8	Linear + carrier	20	14
1-8	Linear + carrier	80	8
S41	Hairpin (2) + carrier	20–40	136
S41	Hairpin (3)	10	18
S41	Hairpin (3)	10	8
S41	Linear + carrier	20	30
S41	Linear + carrier	80	51

Each entry represents a different transfection of the indicated cell line. Chloramphenicol-resistant (Cam^r) colonies were obtained upon transformation of *E. coli* with 1/50th of the reisolated transfected material. Transfections of the two cell lines with DNA preparation 3 were performed in parallel. Preparation numbers are in parentheses.

*Total amount of DNA in samples with added carrier was 150 ng. Amount of hairpin DNA in preparations 1 and 2 was estimated to be within the ranges shown. The 7.7-kb conventional linear fragment was prepared as described for the first step of the hairpin preparation in *Materials and Methods*.

†Numbers of Cam^r colonies (raw counts) are shown. All transformations were done in parallel with control DNA; uptake efficiency was 2.0–2.5 × 10^9 transformants per μg of pUC19.

Further characterization revealed that most junctions (listed above dashed lines in Fig. 3) contained at least one full-length end. Significantly, 32 of the 44 full-length ends (73%) possessed attached P inserts (Fig. 3, boldface; data not shown). A direct implication of this result is that in a large number of cases the hairpin ends were opened through the introduction of a single-strand nick near the terminus. Moreover, while some of the full-length termini lacked adjacent P nucleotides, and as such might have been formed by nicking the hairpin structure at the exact tip, those with P insertions must have been cut open at a location away from the exact apex. The left end, for example, was nicked on one strand at a position at least 5 residues in from the tip in four independent isolates (Fig. 3, HPS-103; data not shown). Thus, while several different routes of hairpin end processing could be envisioned (see *Discussion*), in actuality, the tested cells metabolized hairpin DNA in a way that gave rise to palindromic junctional insertions.

Hairpin resolution junctions isolated from either wild-type or *scid* lymphoid cells were compared as shown in Fig. 3 *A* and *B*. There were no conspicuous differences in the frequency and length of P insertions or the degree of end truncation (Table 2 and Fig. 3). The similarity in hairpin end processing by the two cell lines was underscored by the fact that several sets of identical junctions were isolated from each (Fig. 4). Moreover, *scid* and wild-type cell lines generated similar numbers of recircularized molecules (Table 1). The concentration of DNA in the transfection mixture was very low; the ratio of DNA molecules to cells was only on the order of ≈400:1, and there were no quantifiable differences between *scid* and wild-type cells whether or not carrier DNA was included (Table 1). This ruled out the possibility that high DNA levels might have obscured a difference in DNA joining proficiency. In short, there was no evidence for a hairpin resolution defect in *scid* cells (Tables 1 and 2; Fig. 3).

Hairpin Resolution in Fibroblastoid Cells. To investigate whether the ability to resolve hairpin DNA ends by nicking near the tips was limited to the lymphoid lineage, the hairpin substrate was transfected into NIH 3T3 fibroblastoid cells.

Table 2. Properties of junctions formed via hairpin end joining

Sample	% Cam^r (n)*	% full-length ends (n)†	Ends with P (n)‡	No. of P ≥ 3 (n)‡	No. of P = 5 (n)§
1-8	58 (59)	44 (18)	11 (14)	4 (14)	2 (14)
S41	72 (57)	56 (18)	16 (19)	7 (19)	2 (19)
NIH 3T3	55 (40)	42 (26)	6 (12)	1	0
All left ends			14 (23)	6 (23)	4 (23)
All right ends			12 (22)	5 (22)	0 (22)

Comparisons of *scid* vs. wild type, lymphoid vs. fibroblastoid, and left end vs. right end are presented. n, Total number of isolates, or ends, analyzed for each determination. Entries in columns 3–6 refer to chloramphenicol-resistant (Cam^r) recircularized products. Column 4 gives the number of full-length ends in each sample that exhibited P addition. In columns 5 and 6, the length of those P inserts is shown.

*Transformants were plated on ampicillin; individual colonies were then picked and streaked on medium containing ampicillin only and ampicillin plus chloramphenicol.

†Full length ends, defined as shown in Fig. 3, expressed as percentage of the total number analyzed.

‡P insertions were scored as described; minimum values are presented. n, Total number of full-length ends analyzed. Where an entire insertion could be considered a P nucleotide in relation to either of two ends (e.g., HPW-136 in Fig. 3) it was not included in the left- vs. right-end comparison.

§No P insertion was (without ambiguity) >5 residues in these experiments.

The fine structure of the hairpin resolution junctions formed in NIH 3T3 cells was very similar to those isolated from the lymphoid cell lines. Some of the junctions were identical to the lymphoid isolates. All such cases possessed 1- or 2-base homologies at the crossover site (underlined in Fig. 4), consistent with suggestions that short homologies between ends may tend to guide both V(D)J joining (for a recent discussion, see ref. 18) as well as end joining in general (19). One difference overall was the absence of N regions in the fibroblast sample (Fig. 3*C*). This was to be expected, based on other studies indicating that N region addition in NIH 3T3 cells is dependent on the introduction of a terminal deoxynucleotidyltransferase expression vector (20). The NIH 3T3 cells appeared to be more proficient at hairpin DNA joining than the lymphoid cells (data not shown), although given that fibroblastoid and lymphoid cells required the use of different transfection protocols, an exact quantification of the difference was not attempted.

Structure of the Hairpin DNA Substrate. A critical consideration for these studies was the construction and integrity of the hairpin substrate. As detailed (Fig. 2; *Materials and Methods*), the hairpin substrate was synthesized in a manner specifically designed to minimize the possibility of contamination with "open-ended" molecules. The initial ligation, which created a phosphodiester bond accounting for the covalent closure at the hairpin termini, was followed by two gel-purification steps, each of which would separate any nonligated material from the desired product. Moreover, the critical phosphodiester bond was required by the step in which fragments were "folded" (see Fig. 2). The two halves of the substrate, bearing preformed hairpin termini, were then assembled by ligation at a site several kilobase pairs removed from either end.

Isolate	LEFT	P	P	RIGHT
HPW-108	GGCTGCAGGTC	GA		CCGGGGAT
HPS-64	GGCTGCAGGTC	GA		CCGGGGAT
HPF-75	GGCTGCAGGTC	GA		CCGGGGAT
HPW-11	GGCTGCAGGTC	GA		CCCCGGGGAT
HPS-25	GGCTGCAGGTC	GA		CCCCGGGGAT
HPF-60	GGCTGCAGGTC	GA		CCCCGGGGAT
HPW-109	GGCTGCAGGTC	GA		TCCCCGGGGAT
HPW-132	GGCTGCAGGTC	GA		TCCCCGGGGAT
HPS-138	GGCTGCAGGTC	GA		TCCCCGGGGAT
HPF-74	GGCTGCAGGTC	GA		TCCCCGGGGAT
HPW-7	GGCTGCAGGTC	GACCT	GA	TCCCCGGGGAT
HPS-103	GGCTGCAGGTC	GACCT	GA	TCCCCGGGGAT

FIG. 4. Sets of identical junctions isolated from wild-type (prefix HPW), *scid* (prefix HPS), and fibroblastoid (prefix HPF) cells. Similar total numbers of recombinants were screened in the lymphoid samples. Sequences are displayed as described in Fig. 3.

Because the assay for hairpin resolution involved bacterial transformation, it was important to investigate the extent to which hairpin end resolution could take place in *E. coli*. When 5 ng of the hairpin linear preparation was introduced directly into bacterial cells, no drug-resistant colonies were recovered. This represented a reduction of at least 5 orders of magnitude relative to results obtained with circular plasmid DNA and 2 orders of magnitude relative to the numbers arising upon introduction of the open linear form of the substrate into bacteria (see *Materials and Methods* for details). Evidently *E. coli* is profoundly incompetent in recircularization of the hairpin linear DNA, providing the basis for the present sensitive tests. Results further summarized in *Materials and Methods* established the purity of the hairpin substrate.

DISCUSSION

Hairpin Resolution and V(D)J Joining. Recircularization of a hairpin linear DNA molecule, barring involvement of an unprecedented joining activity, requires removal of the terminal 5′-to-3′ phosphodiester linkage. This could be accomplished in any of several ways: (*i*) by breaking both strands of the linear DNA at some unspecified distance from the terminus, (*ii*) by direct hydrolysis of the terminal closure, or (*iii*) by introduction of a nick near the tip. It is important to note that only the last of these possibilities, the introduction of a single-strand nick near, but not directly at, the hairpin tip, would create the single-strand extension necessary for generation of a P nucleotide insertion.

Vertebrate cells are proficient at connecting both cohesive and noncohesive DNA ends, but the exact steps involved in this process are not yet fully defined (reviewed in ref. 21). Postulated operations include alignment of ends (22), replication of 3′ (and 5′) overhangs, trimming, and ligation. Despite the uncertainty surrounding the steps that intervene between the creation of a conventional terminus from a hairpin end, and the incorporation of this end into a junction, the presence of P nucleotides in the final recombinant provides two definitive pieces of information. First, for every example of P insertion, the single-strand nick pathway is established as the mode of hairpin removal; second, the length of the P insertion itself indicates the closest position relative to the apex of the hairpin end at which this single-strand nick could have been introduced.

The present result, that junctions with P nucleotide insertions were created upon resolution of a hairpin precursor in murine cells, supports the hypothesis that there may be a hairpin intermediate in V(D)J joining (5, 8, 9). Not only has it been shown that a hairpin molecule can be metabolized in these cells, but the details of hairpin processing are exactly

as mandated by the hairpin model (8). A cell-free V(D)J joining system is not yet available, so that it is not possible to isolate a hairpin V(D)J recombination intermediate and chase it into product; however, the present study provides a critical link, demonstrating the creation of palindromic insertions in junctions from a hairpin DNA precursor.

The detection of hairpin resolution activity in fibroblastoid cells indicates that the ability to nick and join hairpin ends is present in cell types outside the lymphoid system and may be an important type of DNA metabolism in some circumstances. The observation that *E. coli* cells are not similarly equipped highlights the fact that the ability to efficiently resolve hairpin DNA is not universal. The significance of hairpin end resolution in mammalian cells, aside from its likely central role in V(D)J joining, remains to be determined.

With regard to antigen receptor gene assembly, although there could conceivably be hairpin end nicking activities other than that revealed in the present study, there is no evidence to indicate this is so. A more precise description of the site(s) of hairpin scission must await biochemical analyses; however, the observed resolution through nicking at positions removed from the exact tip suggests a specialized activity. Moreover, most of the junctions formed in the wild-type lymphoid cells shown in Fig. 3 are structurally indistinguishable from junctions created by site-specific recombination of V(D)J joining substrates in the same cell line (5). This similarity extends as well to the hairpin resolution junctions and V(D)J joining products created in fibroblastoid cells (Fig. 3*C*; ref. 20).

Parallels between nonspecific end-joining products and V(D)J junctions, solidified by the present study, have been noted (19). Here, all features of V(D)J coding joints have been reconstructed: deletions, insertions, and P nucleotides were present in most isolates. In addition, ends were often found to have become connected at 1- or 2-bp homologies (Figs. 3 and 4, underlining). These consistent similarities signify a possible overlap between activities involved in antigen receptor gene assembly and DNA repair (reviewed in ref. 1). One implication of the ability of NIH 3T3 cells to resolve hairpin ends is that RAG-1 and RAG-2 gene products (normally absent from NIH 3T3 cells; refs. 23 and 24) are unlikely to be critically involved in hairpin nicking.

The Nature of the *scid* Defect: A Suggestion. The *scid* mutation affects not only V(D)J joining but also end joining in general (refs. 10 and 11; reviewed in ref. 1). In the present study, the notion that the *scid* mutation interferes specifically with hairpin DNA metabolism (9) was investigated. The results shown here indicate that *scid* cells possess an active hairpin endonuclease.

Although results weigh against a specific hairpin-processing defect in *scid*, end joining is clearly not normal in this background. End-joining deficiencies have been most obvious in experiments in which chromosomal breaks were monitored. Repair of damage induced by ionizing radiation or bleomycin (11–13), integration of foreign DNA into the chromosome (25), and rejoining of chromosomal breaks generated upon electroporation of restriction enzymes into *scid* cells (26) are all altered relative to wild-type cells. Curiously, recircularization of extrachromosomal DNA is affected much less, if at all (ref. 25; this study). An inference suggested by these observations is that while the enzymatic machinery involved in end joining is spared by the *scid* mutation, possibly the *scid* mutation interferes nonenzymatically with certain types of end-to-end interactions. It is conceivable that a facilitated type of encounter is involved in the rejoining of chromosomal breaks [and in the formation of all V(D)J junctions] but that, in extrachromosomal recircularization experiments, physical linkage of the ends instead ensures a reasonable probability of random collision.

In this regard, one subtle difference between hairpin resolution junctions and normal V(D)J joints may be significant. Some of the P insertions are longer in hairpin resolution junctions than is observed in V(D)J junctions (Fig. 3; ref. 5). According to a large survey of ≈1000 full-length ends within published endogenous V(D)J junctions, as well as of 200 full-length ends incorporated into the junctions formed on an artificial V(D)J joining construct, even 4-residue stretches of P nucleotide sequence are extremely rare (5). In the relatively small collection of hairpin resolution junctions available here, several unambiguous examples of contiguous 5-residue P insertions were found (Table 2). Notably, long P insertions are characteristic of the very infrequent V(D)J junctions formed in *scid* cells (reviewed in ref. 1). Perhaps the similarity between the hairpin recircularization junctions and the structures of rare endogenous V(D)J joints formed in *scid* animals is a clue indicating that both types of junction are created in the absence of some structural, stabilizing factor.

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